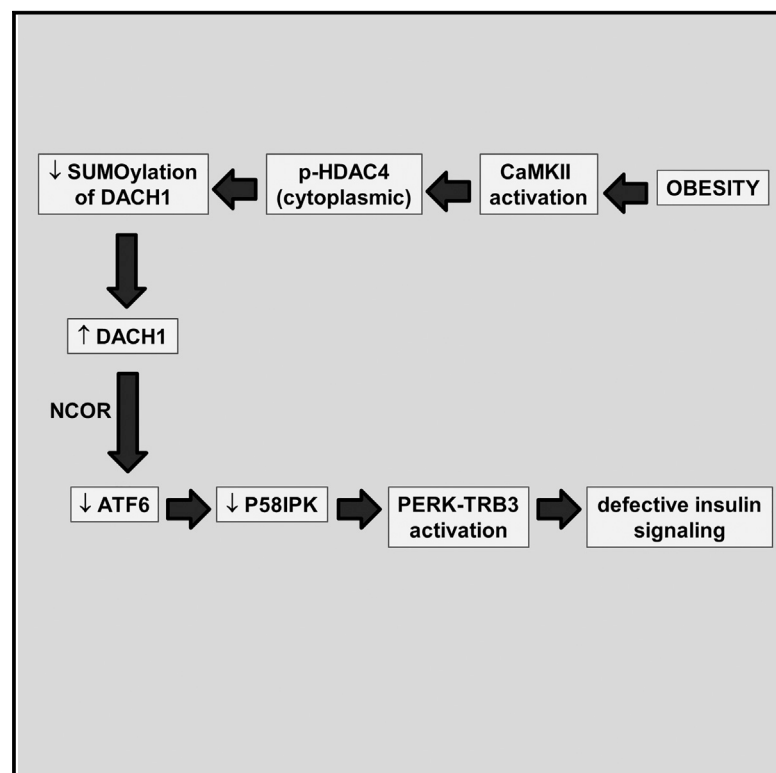


Hepatocyte DACH1 Is Increased in Obesity via Nuclear Exclusion of HDAC4 and Promotes Hepatic Insulin Resistance

Graphical Abstract



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In Brief

Ozcan et al. find that CaMKII phosphorylates and blocks nuclear translocation of hepatocyte HDAC4 in obesity. Lower nuclear HDAC4 decreases the SUMOylation and degradation of the corepressor DACH1. Elevated nuclear DACH1 activates an ER stress pathway that causes defective insulin signaling, while silencing of hepatocyte DACH1 in obesity improves insulin sensitivity.

Highlights

- In obesity, CaMKII phosphorylates and blocks HDAC4 nuclear translocation in hepatocytes
- Lower nuclear HDAC4 decreases the SUMOylation and degradation of DACH1
- Increased nuclear DACH1 represses *Atf6*, which causes defective insulin signaling
- Silencing of hepatocyte DACH1 in obesity improves insulin sensitivity



Hepatocyte DACH1 Is Increased in Obesity via Nuclear Exclusion of HDAC4 and Promotes Hepatic Insulin Resistance

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SUMMARY

Defective insulin signaling in hepatocytes is a key factor in type 2 diabetes. In obesity, activation of calcium/calmodulin-dependent protein kinase II (CaMKII) in hepatocytes suppresses ATF6, which triggers a PERK-ATF4-TRB3 pathway that disrupts insulin signaling. Elucidating how CaMKII suppresses ATF6 is therefore essential to understanding this insulin resistance pathway. We show that CaMKII phosphorylates and blocks nuclear translocation of histone deacetylase 4 (HDAC4). As a result, HDAC4-mediated SUMOylation of the corepressor DACH1 is decreased, which protects DACH1 from proteasomal degradation. DACH1, together with nuclear receptor corepressor (NCOR), represses *Atf6* transcription, leading to activation of the PERK-TRB3 pathway and defective insulin signaling. DACH1 is increased in the livers of obese mice and humans, and treatment of obese mice with liver-targeted constitutively nuclear HDAC4 or DACH1 small hairpin RNA (shRNA) increases ATF6, improves hepatocyte insulin signaling, and protects against hyperglycemia and hyperinsulinemia. Thus, DACH1-mediated corepression in hepatocytes emerges as an important link between obesity and insulin resistance.

INTRODUCTION

Excessive hepatic glucose production (HGP) and aberrant insulin receptor signaling are the major causes of hyperglycemia and insulin resistance in obesity-induced type 2 diabetes (T2D) (Pajvani and Accili, 2015). We have recently identified activation of hepatocyte (HC) CaMKII/p38 as a major contributor to aberrant HGP and perturbed insulin signaling in obesity (Ozcan et al., 2012, 2013). Treatment of obese mice with a drug inhibitor of the pathway lowers plasma glucose and corrects hyperinsuline-

mia and is additive in these benefits with the current leading T2D drug, metformin (Ozcan et al., 2015). The CaMKII pathway in HCs perturbs metabolism by lowering the transcription factor ATF6, which can function as a homeostatic regulator of the endoplasmic reticulum stress response by inducing the chaperone, p58^{IPK} (Wu et al., 2007). As such, the decrease in ATF6 in obese HCs activates the PERK-ATF4 arm of the ER stress response, which induces an inhibitor of insulin receptor signaling called TRB3. In support of the key role of HC ATF6 in the CaMKII pathway as a driver of perturbed metabolism in obesity, both total and nuclear (cleaved) ATF6 are decreased in obese mice liver, and in human liver, ATF6 cleavage decreases in relation to homeostatic model assessment of insulin resistance (HOMA-IR) (Kumashiro et al., 2011; Wang et al., 2009). Moreover, ATF6 overexpression in cell culture models improves insulin signaling, although the underlying mechanisms have not been fully explored (Tang et al., 2011; Ye et al., 2010). ATF6 can also suppress HGP by disrupting the interaction of two gluconeogenic transcription factors, CREB and CRTC2, and restoration of ATF6 levels in the livers of obese mice lowers blood glucose (Wang et al., 2009).

Given the importance of CaMKII-mediated ATF6 suppression in HCs in linking obesity to T2D, a key question relates to the molecular mechanism through which CaMKII suppresses ATF6. We show here that the corepressor DACH1 is responsible for suppressing *Atf6*. DACH1 levels are increased in obesity by CaMKII-induced nuclear exclusion of HDAC4, which decreases HDAC4-mediated SUMOylation and degradation of DACH1. Accordingly, insulin sensitivity can be improved in obese mice by silencing either CaMKII or DACH1 or enforcing nuclear HDAC4 in HCs, without any change in food intake or body weight. These results identify DACH1 as a critical component of defective insulin action seen in obesity.

RESULTS

ATF6 Overexpression in Obese Mice Improves Insulin Sensitivity

While our previous work implicated suppression of ATF6 as a key mechanism of how CaMKII activation in HCs promotes insulin

resistance in obesity (Ozcan et al., 2013), we asked whether genetically boosting hepatic ATF6 in obesity could improve metabolism. To this end, we treated diet-induced obese (DIO) mice with adenoviral vectors encoding cleaved, nuclear ATF6 (ATF6N) or LacZ control. Adeno-ATF6N-treated mice had lower fasting blood glucose and lower plasma insulin levels and enhanced reduction of blood glucose in response to insulin stimulation (Figures S1A and S1B) in the absence of any change in body weight. Adeno-ATF6N treatment also improved acute insulin-induced p-Akt (Figure S1C), which is a dynamic measure of improved insulin signaling.

CaMKII Suppresses HC *Atf6* mRNA in Obesity

Given the critical role of ATF6 in CaMKII-mediated improvement in insulin signaling, we next investigated whether CaMKII regulates ATF6 at the transcriptional level. For this purpose, we first analyzed the livers of *Camk2g*^{fl/fl} obese mice treated with control AAV-TBG-LacZ versus AAV-TBG-Cre, which specifically deletes CaMKII in HCs and thereby increases insulin sensitivity (Ozcan et al., 2013). The livers of obese mice with HC-deleted CaMKII had higher *Atf6* mRNA levels than the livers of the control obese mice (Figure S1D). We then isolated primary HCs from *Camk2g*^{fl/fl} mice and treated them ex vivo with adeno-Cre to delete CaMKII or with adeno-LacZ control, followed by incubation with BSA (control) or palmitate. Palmitate mimics features of HC pathophysiology seen in obesity (Boden et al., 2005), including defective insulin signaling (Ozcan et al., 2013). We first confirmed the increase in *Atf6* mRNA in palmitate-treated CaMKII-deficient HCs (Figure S1E) and then conducted *Atf6* promoter chromatin immunoprecipitation (ChIP) assays. CaMKII-deficient HCs showed increased *Atf6* promoter occupancy of both RNA polymerase II (Pol II) and H3K27ac, a histone mark associated with active gene transcription (Wang et al., 2008) (Figure S1F). In summary, hepatic CaMKII activation in obese mice suppresses *Atf6* transcription, and restoring hepatic ATF6 through genetic engineering improves metabolic health.

Obesity Induces HDAC4 Phosphorylation and Regulates Its Nuclear Localization

The data above raise the key issue of how hepatic CaMKII lowers *Atf6* transcription in obesity. Previous work has implicated CaMKII in transcriptional regulation through its ability to phosphorylate and promote the nuclear export of the class IIa histone deacetylase HDAC4 (Backs et al., 2006; Zhang et al., 2007). The possible relevance of CaMKII-induced phosphorylation and nuclear export of HDAC4 in obese mouse liver was suggested by the results of several experiments. First, phosphorylation of HDAC4 on Ser465 and Ser629 (corresponding to Ser467 and Ser632 in human HDAC4), two CaMKII phosphorylation sites (Backs et al., 2006), was significantly increased in the livers of obese mice compared with lean mice (Figure 1A). Second, in the livers of obese mice, nuclear HDAC4 levels were lower and cytoplasmic levels were higher (Figure 1B). Third and most importantly, liver-CaMKII γ deficiency or CaMKII inhibition markedly decreased p-Ser465 and p-Ser629-HDAC4 and promoted HDAC4 nuclear localization in the livers of obese mice (Figures 1C and 1D). Palmitate-treated CaMKII-deficient HCs also showed increased nuclear HDAC4 compared to HCs from control mice (Figure 1E). To determine causation, we silenced

HDAC4 in CaMKII-deficient HCs using small interfering RNA (siRNA) and found that this lowered *Atf6* and its downstream target, *Dnajc3* (P58^{IPK}), and abrogated the improvement in insulin-Akt signaling in CaMKII-deficient HCs upon palmitate treatment (Figures 1F and 1G). We then tested causation in vivo by silencing HDAC4 in DIO mice and found increased fasting blood glucose and plasma insulin levels and reduced glucose-disposal curves post-insulin (Figure S2) without a change in body weight. These data provide initial support for the role of an obesity-driven CaMKII-HDAC4 nuclear exclusion pathway in ATF6 suppression and defective insulin signaling in HCs.

To further establish causation, we took advantage of a phosphorylation-defective, constitutively nuclear HDAC4 mutant, HDAC4S3A (S246A/S467A/S632A) (Backs et al., 2006). Transfection of primary HCs with a relatively low titer of this mutant increased *Atf6* and *Dnajc3* mRNA levels and improved insulin-induced p-Akt after palmitate treatment (Figures 2A and 2B; compare white and black bars). To test the functional role of ATF6 in this improvement, we silenced *Atf6* in palmitate-treated HDAC4S3A-overexpressing HCs using siRNA and found that this treatment lowered *Dnajc3* and reduced insulin-induced p-Akt to the level of palmitate-treated control HCs (Figures 2A and 2B; compare black and gray bars). We then treated DIO mice with adenoviral vectors encoding the mutant HDAC4 versus LacZ control. Adeno-HDAC4S3A-treated mice had lower fasting blood glucose and lower plasma insulin levels in the absence of any change in body weight (Figures 2C and 2D). To link these findings to hepatic insulin signaling, we assayed p-Akt in the livers of mice injected with insulin through the portal vein. Similar to the case with palmitate-treated HCs, there was enhanced insulin-stimulated Akt phosphorylation in the livers of adeno-HDAC4S3A-treated DIO mice (Figure 2E). Finally, acetylation of the key gluconeogenic transcription factor FoxO1, which suppresses its binding to DNA target sites, was shown to be increased in HCs and in the liver of lean mice with combined silencing of HDAC4, HDAC5, and HDAC7 (Mihaylova et al., 2011). However, we found that when hepatic HDAC4 alone was either silenced or rendered constitutively nuclear (HDAC4S3A) in obese mice, the level of acetylated FoxO1 in the liver was not altered (Figure S2F). These collective data indicate the presence of a CaMKII \rightarrow HDAC4 phosphorylation/nuclear exclusion \rightarrow ATF6 suppression pathway in obese liver, resulting in perturbed hepatic insulin signaling and disturbed glucose homeostasis.

Nuclear Exclusion of HC HDAC4 in Obesity Elevates Nuclear DACH1, a Corepressor that Lowers *Atf6* and Promotes Insulin Resistance

We next turned our attention to the mechanism of how HDAC4 could induce *Atf6* transcription. Because HDAC4 usually represses gene transcription (Parra, 2015), we considered the possibility that HDAC4 repressed an *Atf6* gene repressor. Indeed, previous studies in skeletal muscle identified a transcriptional repressor called Dachshund homolog (DACH) as a target of HDAC4-mediated repression (Tang and Goldman, 2006; Tang et al., 2009). Thus, we hypothesized that nuclear exclusion of HDAC4 in HCs in obesity would result in the de-repression of DACH1, which would then repress *Atf6*. We first asked whether hepatic DACH1 is increased in obesity. DACH1 levels were

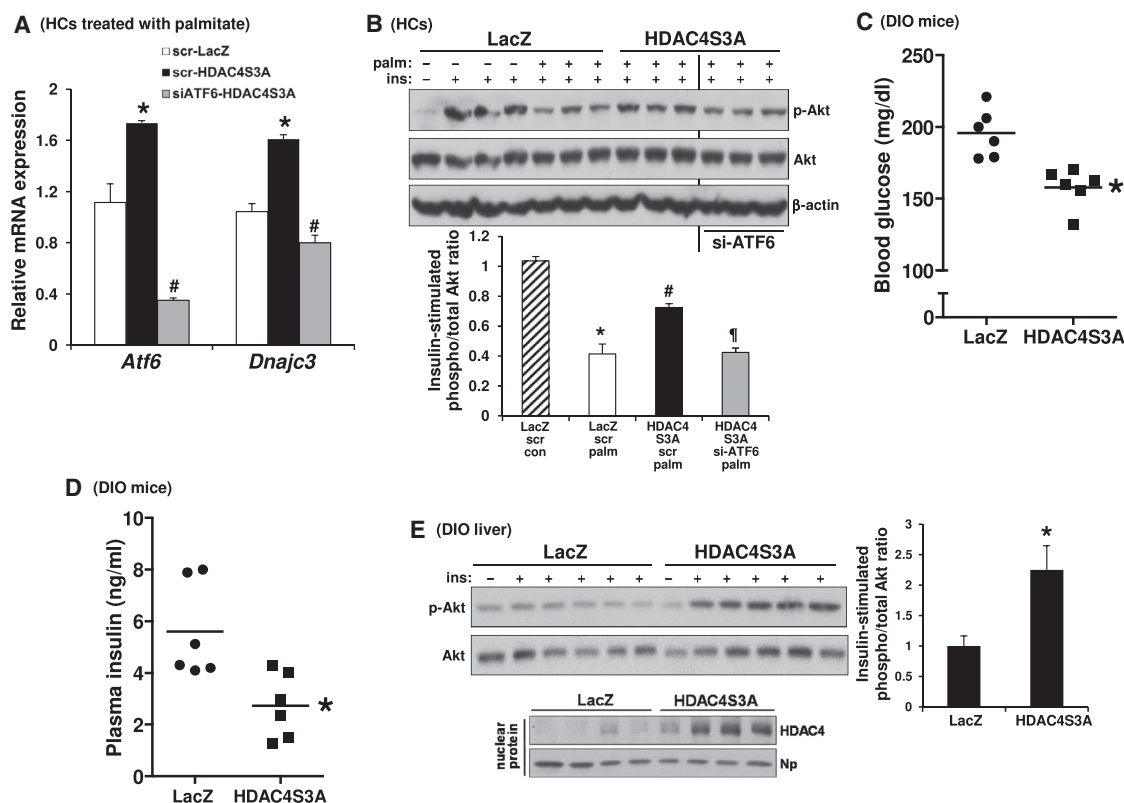


Figure 2. Constitutively Nuclear HDAC4S3A Increases *Atf6*, *Dnajc3*, and Insulin-Stimulated p-Akt and Improves Metabolism in DIO Mice

(A) Primary HCs from wild-type (WT) mice were pretreated with either scrambled RNA (scr) or siRNA targeting ATF6 (siATF6) for 12 hr followed by transduction with adeno-LacZ or adeno-HDAC4S3A. After an additional 24 hr, the cells were incubated with 0.3 mM palmitate for 11 hr, with the last 5 hr in serum-free media. The cells were then assayed for *Atf6* and *Dnajc3* mRNA by qRT-PCR (n = 3; bars with different symbols are different from each other and control, mean \pm SEM, $p < 0.05$). (B) As in (A), except that some of the cells received BSA control instead of palmitate and were then treated with PBS control or 100 nM insulin for 5 min, as indicated by the minus and plus symbols. Cell lysates were then assayed by immunoblot for p-Akt, total Akt and β -actin. Densitometric quantification of the immunoblot data is shown in the bar graph (n = 3; bars with different symbols are different from each other and control, mean \pm SEM, $p < 0.05$). (C and D) Fasting blood glucose and plasma insulin levels in DIO mice treated with adeno-LacZ or adeno-HDAC4S3A (n = 6 mice/group; mean \pm SEM, * $p < 0.05$). (E) DIO mice treated with adeno-LacZ or adeno-HDAC4S3A were fasted for 5 hr and then injected with 1.5 IU/kg insulin through the portal vein for 3 min. Liver extracts were assayed for p-Akt and total Akt (top blot), with densitometric quantification of the data shown in the bar graph (n = 5; mean \pm SEM, * $p < 0.05$). The lower blot shows the expression level of nuclear HDAC4 in the two groups of mice.

See also Figure S2.

HDAC4 in obese mice further increased hepatic DACH1, suggesting that DACH1 lies downstream of HDAC4 (Figure 3C, bottom). We also tested the effect of CaMKII inhibition in metabolism-qualified primary human HCs and found that DACH1 was increased by palmitate treatment and that this increase was prevented by inhibition of CaMKII using adeno-K43A-CaMKII (Figure S3C). Furthermore, analysis of 14 human liver biopsy specimens spanning body-mass indexes (BMIs) from 19 to 62 showed an overall trend for higher levels of DACH1 and p-Ser632-HDAC4 in the livers of obese versus leaner subjects (Figure 3D).

We next tested the importance of DACH1 in the regulation of the ATF6-TRB3-insulin signaling pathway using wild-type (WT) versus DACH1-deficient HCs treated with or without palmitate. For this experiment, we used primary HCs isolated from *Dach1*^{fl/fl} mice and then treated the cells ex vivo with adeno-Cre or control adeno-LacZ. The data show that *Atf6* mRNA was significantly higher and *Trb3* mRNA was significantly lower

in DACH1-deficient HCs (Figure 3E). Most importantly, DACH1 deficiency resulted in prevention of palmitate-induced suppression of insulin-induced p-Akt (Figure 3F).

To test the functional importance of DACH1 in glucose metabolism in obesity, we silenced hepatic DACH1 in DIO mice using adeno-associated virus-8 (AAV8)-mediated small hairpin RNA (shRNA) (Lisowski et al., 2014). This treatment, which lowered hepatic DACH1 levels by ~70% (Figure 4D) without a change in body weight, significantly lowered fasting blood glucose and plasma insulin (Figure 4A). DACH1 silencing also lowered plasma glucose in response to pyruvate challenge, which is a measure of HGP; improved the blood glucose response to glucose challenge; and enhanced reduction of blood glucose in response to insulin stimulation (Figures 4B and 4C). Consistent with our in vitro data, we observed increases in both *Atf6* mRNA and insulin-stimulated Akt phosphorylation and a significant decrease in *Trb3* mRNA levels in the DACH1-silenced DIO cohort (Figures 4D and 4E). Similar results were found in DIO *Dach1*^{fl/fl} mice

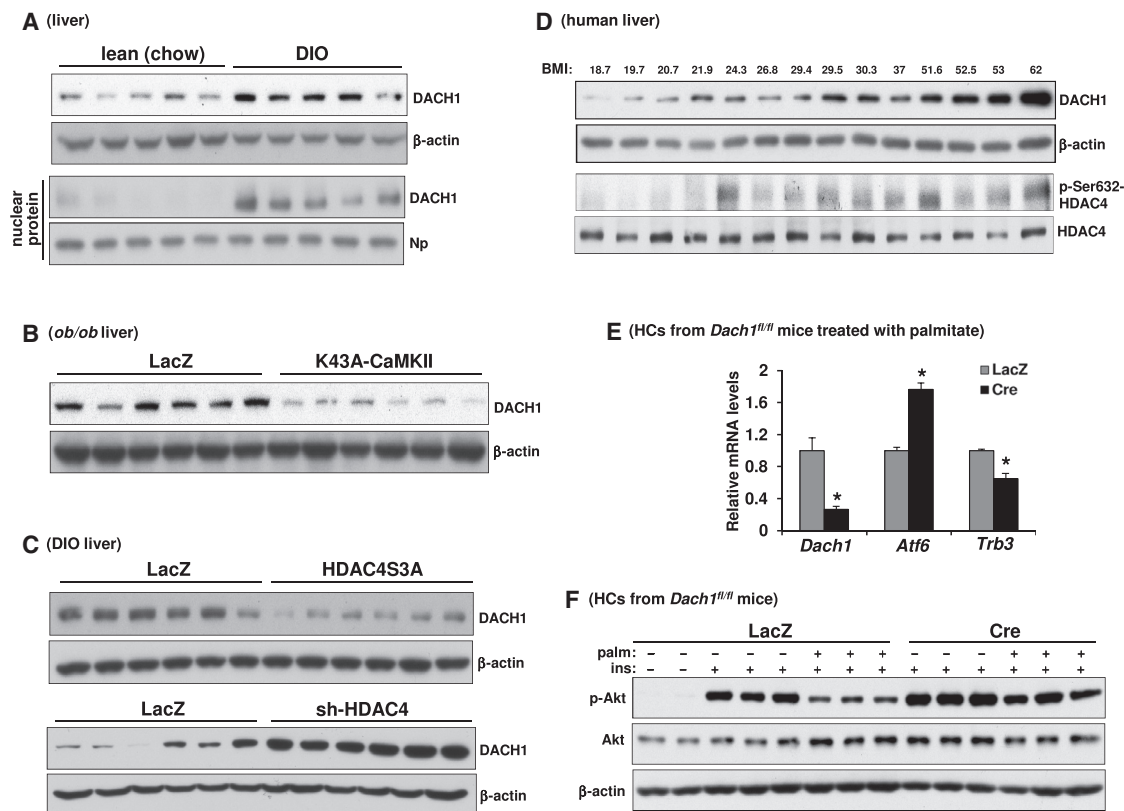


Figure 3. Hepatic DACH1 Is Increased in Obesity via CaMKII Activation and Nuclear Exclusion of HDAC4, Leading to Defective Insulin Signaling in HCs

(A) Extracts of total liver (top blot) and liver nuclei (bottom blot) from WT mice fed a chow or DIO diet for 12 weeks were assayed for DACH1, β-actin, and nucleophosmin (Np) by immunoblot.

(B) DACH1 and β-actin were assayed in liver extracts from *ob/ob* mice treated with adeno-LacZ or adeno-K43A-CaMKII.

(C) DACH1 and β-actin were assayed in livers from DIO mice treated with adeno-LacZ or adeno-HDAC4S3A (top blot) or DIO mice treated with adeno-LacZ or adeno-sh-HDAC4 (bottom blot).

(D) DACH1, β-actin, p-Ser632-HDAC4 and HDAC4 were assayed in liver extracts from human subjects with the indicated BMIs.

(E) HCs from *Dach1^{fl/fl}* mice were transduced with adeno-LacZ or adeno-Cre. After 36 hr, cells were incubated with 0.3 mM palmitate for 11 hr, with the last 5 hr in serum-free media. Cells were then assayed for *Dach1*, *Atf6*, and *Trb3* mRNA ($n = 3$; mean \pm SEM, $*p < 0.05$).

(F) As in (E), except that some of the cells received BSA control instead of palmitate and were then treated with PBS control or 100 nM insulin for 5 min, as indicated by the minus and plus symbols. Cell lysates were then assayed for p-Akt, total Akt, and β-actin.

See also Figure S3.

injected with AAV-8 encoding Cre recombinase driven by the HC-specific thyroxine binding globulin promoter (TBG-Cre) (Figure S4). These data are consistent with the hypothesis that hepatic DACH1 depletion improves glucose metabolism in obese mice by increasing hepatic insulin sensitivity.

DACH1 has a highly conserved N-terminal DACH Box domain, which shares ~35% amino acid identity to the Ski/Sno proteins and is therefore known as the DACH Ski/Sno (DS) domain (Li et al., 2002; Wu et al., 2003). The DS domain mediates the interaction of DACH1 with both DNA and other corepressors and is required for the ability of DACH1 to repress a number of genes (Sundaram et al., 2008; Wu et al., 2003, 2008). Accordingly, an engineered mutant of DACH1 in which the DS domain is deleted (Δ DS-DACH1) has been shown to act in a dominant-negative manner in blocking DACH1 gene repression (Sunde et al., 2006). As a further test of the importance of DACH1 in liver meta-

bolism, we treated obese mice with adeno- Δ DS-DACH1 versus adeno-LacZ control. The Δ DS-DACH1 cohort had lower fasting blood glucose, lower plasma insulin levels, improved blood glucose response to glucose challenge, and enhanced reduction of blood glucose upon insulin stimulation, all without a change in body weight (Figures S5A–S5D). Adeno- Δ DS-DACH1 treatment also improved insulin-induced p-Akt and raised the level of P58^{IPK} (Figure S5E). Similar results were obtained using *ob/ob* mice (Figure S6). These results further support the role of DACH1 in glucose homeostasis in obesity and indicate that the DS domain of DACH1 is required for this effect.

DACH1 Silencing Improves Glucose Metabolism in Obese Mice by Increasing ATF6

To test whether the metabolic improvement seen with hepatic DACH1 deficiency was dependent on the increase in ATF6, we

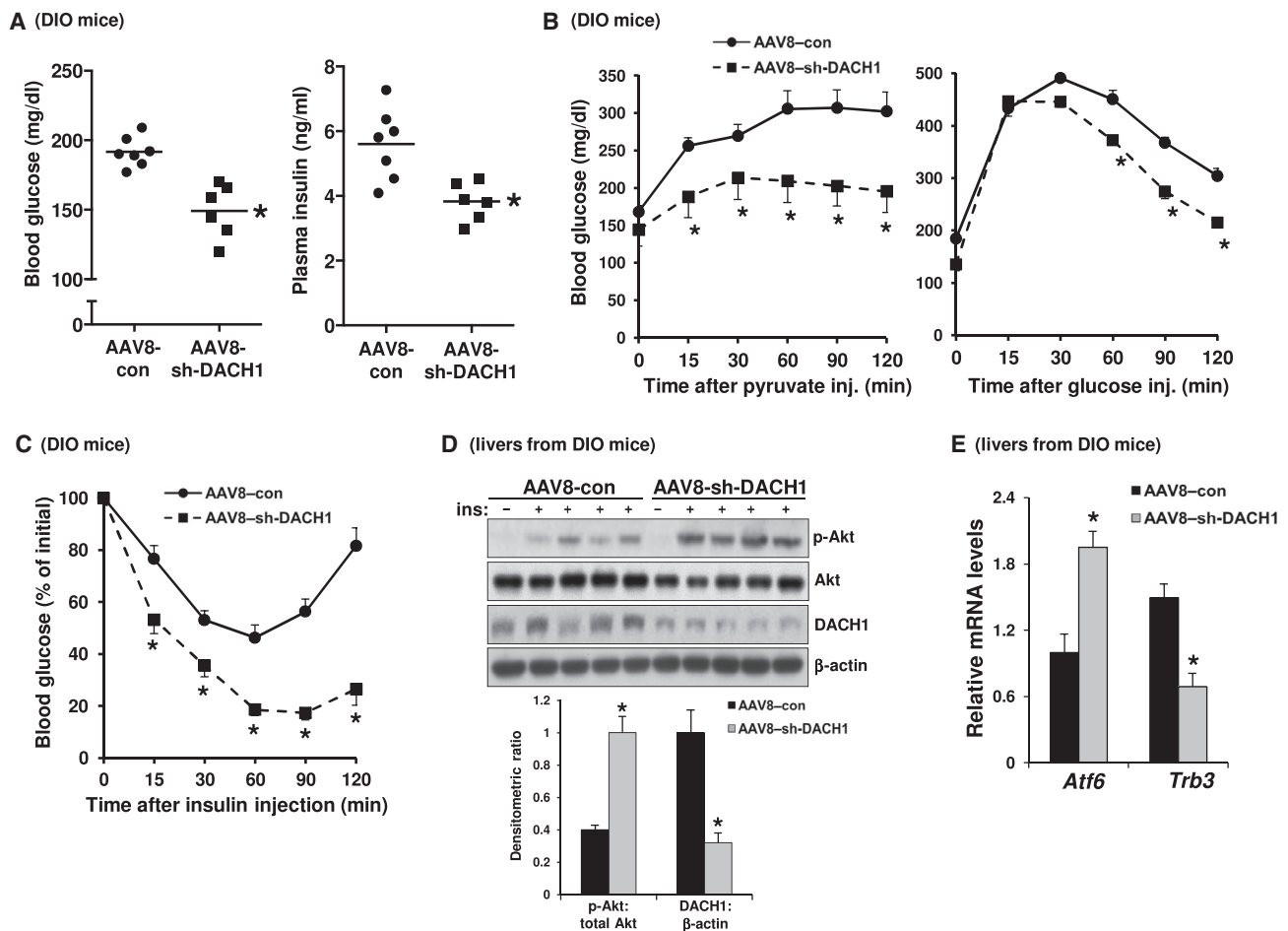


Figure 4. Silencing Hepatic DACH1 in Obese Mice Improves Glucose and Insulin Metabolism

(A–C) Fasting blood glucose and plasma insulin levels (A) and pyruvate, glucose, and insulin tolerance tests (B and C) in DIO mice after treatment with AAV8-sh-DACH1 or empty AAV (AAV8-con) (n = 6–7 mice/group; mean \pm SEM, *p < 0.05).

(D) DIO mice were treated with AAV8-sh-DACH1 or empty AAV (AAV8-con). After 14 days, the mice were fasted for 5 hr and injected with insulin through the portal vein, followed 3 min later by harvesting of liver. Liver extracts were assayed for p-Akt, total Akt, DACH1, and β -actin by immunoblot. Densitometric quantification of the immunoblot data is shown in the graph (n = 4–5; mean \pm SEM, *p < 0.05).

(E) As in (D), except that *Atf6* and *Trb3* mRNA levels were assayed by qRT-PCR (n = 5; mean \pm SEM, *p < 0.05).

See also Figures S4–S6.

silenced hepatic ATF6 in liver-DACH1-deficient obese mice using adeno-sh-ATF6 (Wang et al., 2009). The results show that all of the beneficial effects of DACH1 silencing—decreased blood glucose and plasma insulin, improved blood glucose response to glucose challenge, enhanced reduction of blood glucose in response to insulin, and increased insulin-induced p-Akt in liver—were abrogated by also silencing hepatic ATF6 (Figures 5A–5D). These data provide further evidence that hepatic DACH1 depletion improves insulin sensitivity and glucose metabolism in obese mice by de-repressing ATF6.

Recent work has identified a consensus DACH1 DNA-binding sequence using genome-wide in silico promoter analysis together with cyclic amplification and selection of targets (Zhou et al., 2010). Using this information, we identified an intronic region (intron 14) and an exon (exon 16) in the *Atf6* gene that contain DACH1 consensus sequences. ChIP analysis in GFP-DACH1-

transfected primary HCs showed significantly increased recruitment of DACH1 to both of these regions (Figures 6A, left graph, and 6B), whereas a control segment at the 3' end of the *Atf6* gene gave no signal (not shown). Moreover, in view of the fact that corepressors bind to enhancer sites to suppress gene transcription, we found through additional ChIP experiments that the intronic site was enriched for two enhancer marks, histone H3 lysine 4 monomethylation (H3K4me1) and H3K27 acetylation (H3K27Ac) (Heintzman et al., 2007) (Figure 6A, middle and right graphs). We also observed significantly increased recruitment of DACH1 to the identified intronic and exonic regions in obese liver compared with lean liver, whereas a control segment (Rplp0) gave no signal (Figure 6C).

In view of previous work showing that DACH1 can interact with nuclear receptor corepressor (NCOR) to repress genes (Wu et al., 2003), we tested the interaction between DACH1

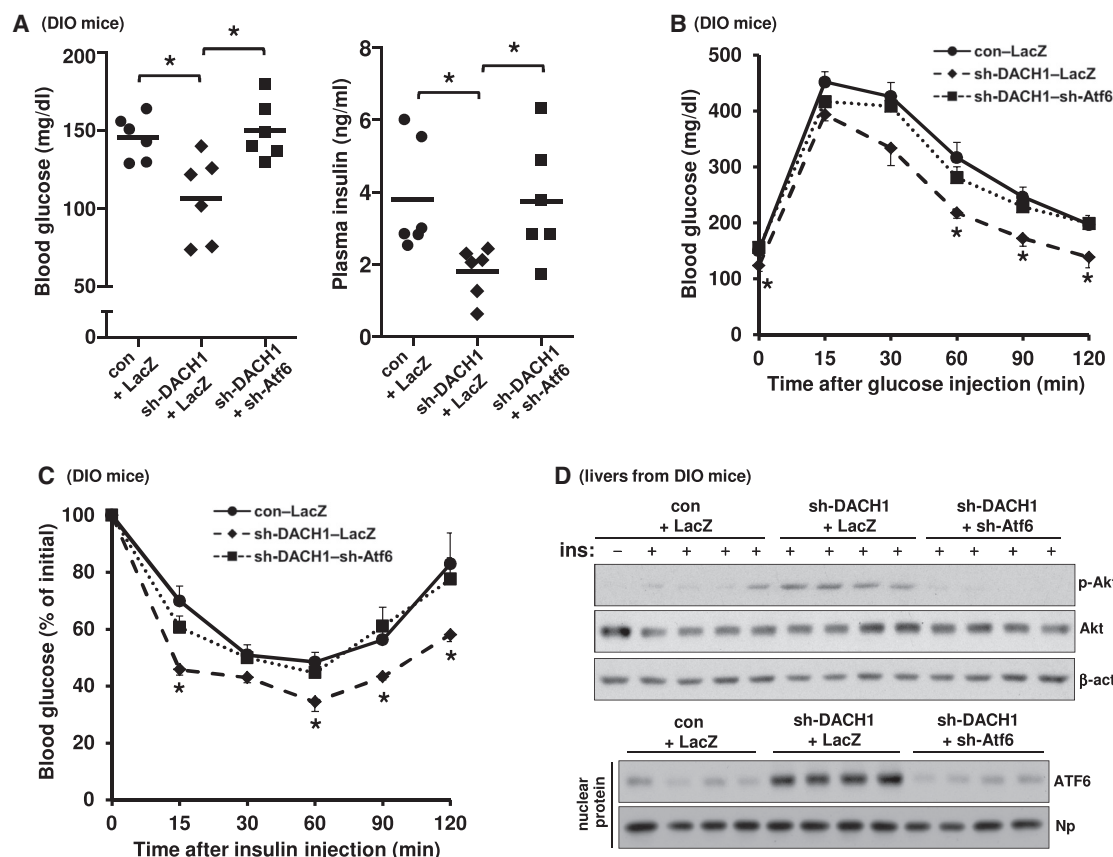


Figure 5. Improvement in Glucose Homeostasis by Hepatic DACH1 Silencing Is Abrogated by Also Silencing ATF6

(A–C) WT DIO mice were treated with AAV8-sh-DACH1 or AAV8-con. After 3 days, half of the AAV8-sh-DACH1 mice received adeno-sh-Atf6, while the other half received adeno-LacZ. After an additional 5 days, fasting blood glucose and plasma insulin levels were assayed, and glucose and insulin tolerance tests were conducted ($n = 6$ mice/group; mean \pm SEM, $p < 0.05$).

(D) WT DIO mice were treated as in (A)–(C). After 14 days, the mice were fasted for 5 hr and injected with insulin through the portal vein, followed 3 min later by harvesting of liver. Total liver extracts were assayed for p-Akt, total Akt, and β -actin (top blot), and nuclear extracts were assayed for ATF6 and nucleophosmin (Np) (bottom blot).

and NCOR. We observed that NCOR could be coimmunoprecipitated with DACH1 in liver from obese, but not lean, mice, suggesting that DACH1 and NCOR physically interact in the setting of obesity (Figure 6D). Consistent with the data in Figure 3A, very little DACH1 could be immunoprecipitated from lean liver. Moreover, ChIP analysis revealed the presence of NCOR on the intronic region where DACH1 binds *Atf6*, and this ChIP signal was markedly diminished in DACH1-deficient cells (Figure 6E). As further evidence, we showed that AAV8-sh-NCOR-treated obese mice had higher hepatic *Atf6* mRNA levels compared with control mice (Figure 6F). These combined data are consistent with a model in which DACH1-NCOR complex binds to DACH1 consensus sites in the *Atf6* gene in hepatocytes in the setting of obesity and represses *Atf6*.

HDAC4-Mediated DACH1 SUMOylation Leads to Its Degradation

To elucidate the molecular mechanism of DACH1 suppression by nuclear HDAC4, we first analyzed the livers of obese mice treated with adeno-LacZ versus adeno-HDAC4S3A and found

no decrease in *Dach1* mRNA in the HDAC4S3A cohort (Figure S7A). Similar results were obtained using palmitate-treated control versus HDAC4S3A-transfected primary HCs (Figure S7B). Using the HC model, we then explored the possibility that nuclear HDAC4 increases the proteasomal degradation of DACH1 protein. Consistent with this idea, the proteasome inhibitor MG132 partially prevented the decrease in DACH1 protein conferred by HDAC4S3A transfection (Figure 7A).

HDAC4 can act as an E3 ligase that affects protein SUMOylation (Grégoire and Yang, 2005; Zhao et al., 2005), which in turn can promote ubiquitination and proteasomal degradation of SUMOylated proteins (Miteva et al., 2010). To address whether a SUMOylation-dependent pathway was involved in the HDAC4-mediated suppression of DACH1, we first asked whether endogenous DACH1 gets SUMOylated. In palmitate-treated HCs, immunoprecipitation and western blot analysis revealed that DACH1 SUMOylation occurs and is diminished upon palmitate treatment, concomitant with an increase in DACH1 protein (Figure 7B). We obtained similar results when we overexpressed DACH1 in HCs (Figures 7C and S7C). Moreover, when we silenced the E2-conjugating enzyme

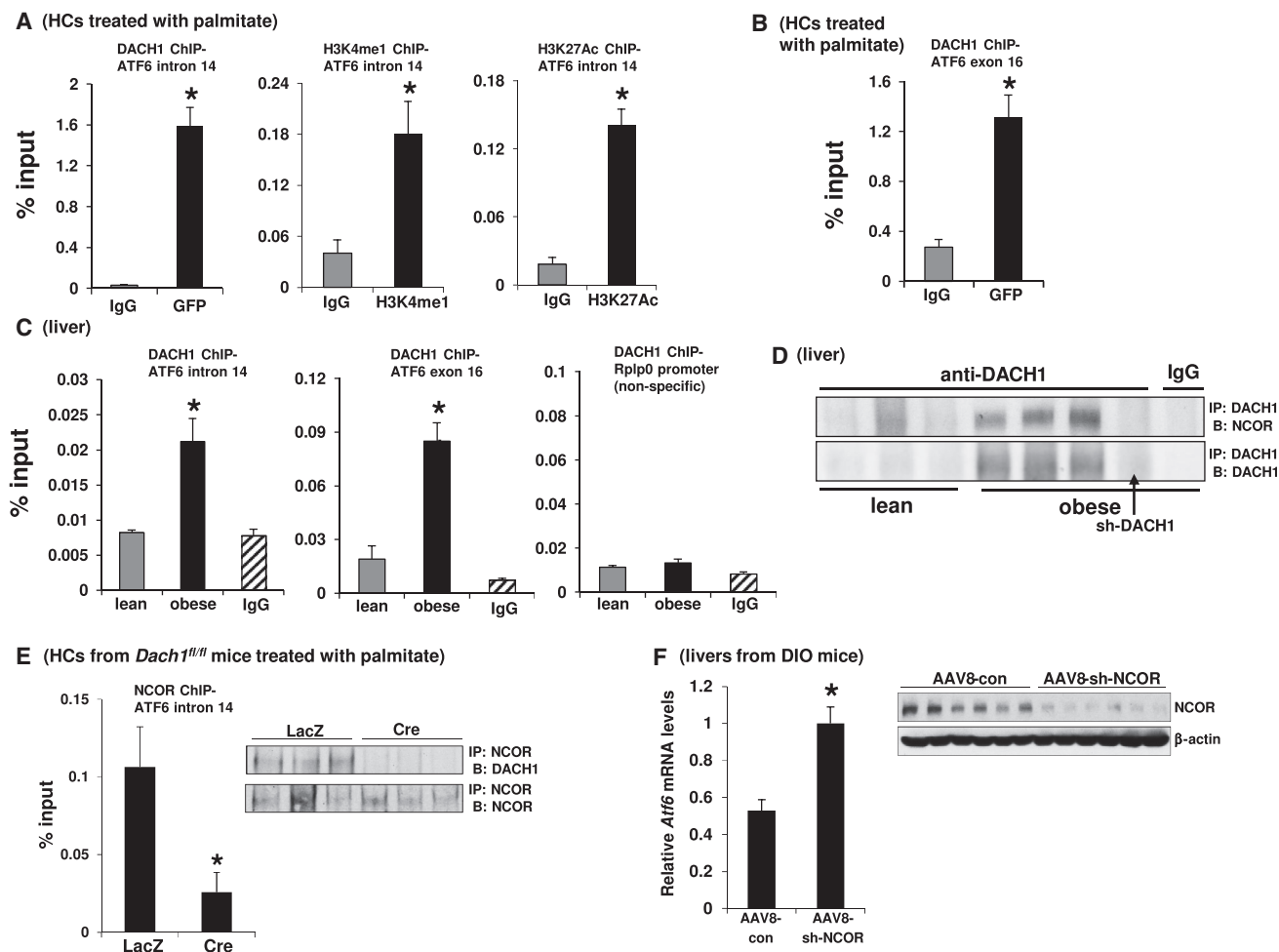


Figure 6. DACH1-NCOR Complex Represses *Atf6*

(A) Primary HCs from WT mice were transfected with an expression plasmid encoding GFP-DACH1. After 48 hr, the cells were incubated with 0.3 mM palmitate for 3 hr, and then ChIP was performed using anti-GFP, anti-H3K4me1, anti-H3K27Ac, or immunoglobulin G (IgG) control antibodies. The region spanning a specific intron site containing a predicted DACH1-binding sequence (intron 14) was amplified by qRT-PCR and normalized to the values obtained from the input DNA ($n = 3$; mean \pm SEM, $*p < 0.05$).

(B) Same as (A), except that region spanning a specific exon site containing a predicted DACH1-binding sequence (exon 16) was amplified by qRT-PCR and normalized to the values obtained from the input DNA ($n = 3$; mean \pm SEM, $*p < 0.05$).

(C) ChIP was performed from liver extracts of lean and DIO mice with anti-DACH1 or IgG control antibodies. The region spanning specific intron and exon sites containing predicted DACH1-binding sequences (intron 14 and exon 16, respectively) and a non-specific region (Rplp0) were amplified by qRT-PCR and normalized to the values obtained from the input DNA ($n = 3$; mean \pm SEM, $*p < 0.05$).

(D) DACH1 was immunoprecipitated (IP) from liver extracts and then probed for NCOR or DACH1 by immunoblot (B). Lanes 1–3 are lean mice, and lanes 4–8 are DIO mice. All DIO mice were treated with AAV8-con, except for lane 7, which was treated with AAV8-sh-DACH1. Lane 8 used control IgG instead of anti-DACH1 for the immunoprecipitation step.

(E) HCs from *Dach1^{fl/fl}* mice were transduced with adeno-LacZ or adeno-Cre. After 48 hr, the cells were incubated with 0.3 mM palmitate for 3 hr, and then ChIP was performed using an antibody against NCOR. The region spanning the aforementioned *Atf6* intronic site was amplified by qRT-PCR and normalized to the values obtained from the input DNA ($n = 3$; mean \pm SEM, $*p < 0.05$). Inset, ChIP from a parallel set of cells that was treated the same as above, up until the final post-immunoprecipitation wash. The beads were then boiled in loading buffer and assayed by immunoblot for NCOR and DACH1.

(F) Liver extracts from DIO mice treated with AAV8-con or AAV8-sh-NCOR were assayed for *Atf6* mRNA levels by qRT-PCR ($n = 6$, mean \pm SEM, $*p < 0.05$). Inset: protein extracts were probed for NCOR and β -actin by immunoblot to document NCOR silencing.

Ubc9, which transfers the activated SUMO to protein substrates (Ghisletti et al., 2007; Hay, 2005), HDAC4-mediated DACH1 degradation was abrogated (Figure 7D). As direct evidence that HDAC4 promotes DACH1 SUMOylation, we found that silencing HDAC4 abrogated DACH1 SUMOylation in primary HCs (Figure 7E).

Based on the consensus SUMO-acceptor site sequence (Rodriguez et al., 2001) (<http://www.abgent.com/sumoplot>), we identified three high-probability sites for SUMOylation in murine DACH1: K341, K644, and K676. In order to determine whether these lysine residues are targets for SUMOylation and degradation of DACH1, we created a mutant of DACH1 with lysine to

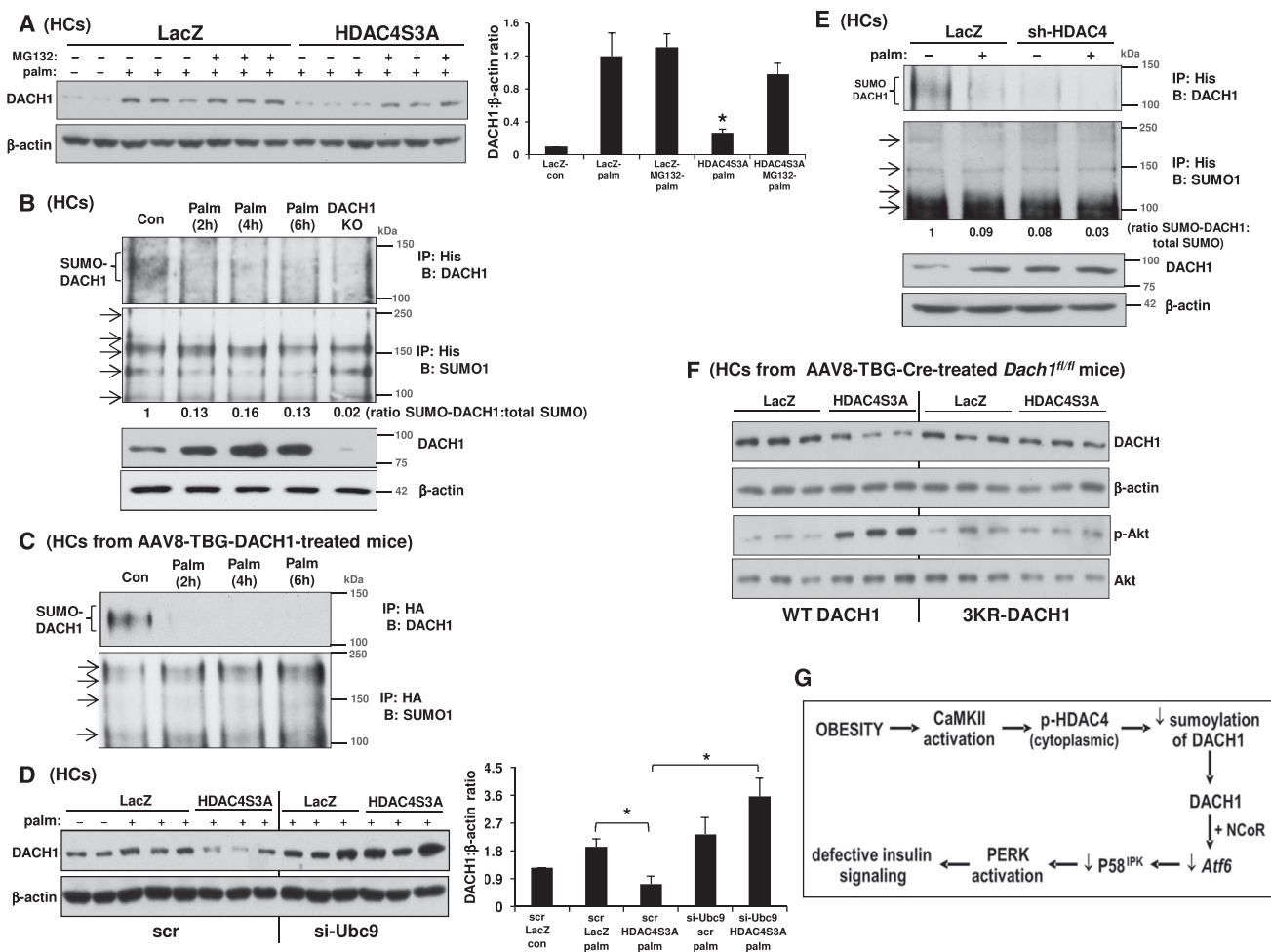


Figure 7. HDAC4-Mediated SUMOylation of DACH1 Leads to Proteasomal Degradation of DACH1 and Defective Insulin Signaling in HCs

(A) Primary HCs from WT mice were transduced with adeno-LacZ or adeno-HDAC4S3A. After 24 hr, the cells were pretreated with either vehicle or MG132 for 1 hr followed by incubation with either BSA or 0.3 mM palmitate (palm) for 6 hr. Lysates were probed for DACH1 and β -actin by immunoblot. Densitometric quantification of the immunoblot data is shown in the bar graph ($n = 3$; mean \pm SEM, $*p < 0.05$ versus all other groups except LacZ-con).

(B) Lysates from WT or DACH1 KO HCs transfected with poly-His-tagged SUMO1 and treated with BSA (Con) or palmitate (palm) were immunoprecipitated using anti-poly-His and blotted for DACH1 or SUMO1. Arrows indicate SUMOylated proteins. The numbers below the SUMO1 immunoblot are the densitometric ratios of SUMO-DACH1:total SUMO. The lower blot shows DACH1 and β -actin in whole cell lysates.

(C) Primary HCs from WT mice treated with AAV8-TBG-DACH1 were transfected with HA-tagged SUMO1 and treated with BSA (Con) or palmitate (palm) as indicated. Lysates were immunoprecipitated using anti-HA and blotted for DACH1 or SUMO1. Arrows indicate SUMOylated proteins.

(D) HCs were pretreated with either scrambled RNA (scr) or siRNA targeting Ubc9 (si-Ubc9). After 12 hr, the cells were transduced with adeno-LacZ or adeno-HDAC4S3A. After an additional 24 hr, the cells were incubated with BSA control or 0.3 mM palmitate (palm) for 5 hr as indicated by the minus and plus symbols. Lysates were then probed for DACH1 and β -actin by immunoblot. Densitometric quantification of the immunoblot data is shown in the bar graph ($n = 3$; mean \pm SEM; $*p < 0.05$; bars 4 and 5 are not significantly different).

(E) Similar to (B), except that the cells were transduced with adeno-LacZ or adeno-sh-HDAC4.

(F) HCs from *Dach1*^{fl/fl} mice treated with AAV-TBG-Cre were transduced with plasmids encoding WT DACH1 or 3KR mutant DACH1. After 12 hr, the cells were transduced with adeno-LacZ or adeno-HDAC4S3A. After an additional 24 hr, the cells were incubated with 0.3 mM palmitate for 11 hr, followed by insulin stimulation for 5 min. Lysates were probed for DACH1, β -actin, p-Akt, and Akt.

(G) Summary scheme of a CaMKII-HDAC4-DACH1 pathway linking obesity to defective insulin signaling in HCs. Based on the data in this report and our previous publications (Ozcan et al., 2012, 2013), obesity-induced activation of CaMKII in HCs phosphorylates HDAC4, which promotes its exit from the nucleus. As a result, nuclear SUMOylation of the corepressor DACH1 is decreased, which prolongs its half-life and increases its level in the nucleus. DACH1, together with NCOR, decreases the transcription of *Atf6*, leading to activation of the PERK-TRB3 pathway and defective insulin signaling, as described previously (Ozcan et al., 2013). See also Figure S7.

arginine substitutions at these three sites (DACH1-3KR mutant) and transfected DACH1-deficient HCs with this construct. In non-palmitate-treated HCs, WT DACH1 was SUMOylated as

before, but this was not seen with DACH1-3KR (Figure S7D). Furthermore, HDAC4S3A was unable to decrease DACH1 levels in the DACH1-3KR-transfected cells, in contrast to the situation

with HCs expressing WT DACH1 (Figure 7F, top two blots). Most importantly, the improvement in insulin signaling conferred by HDAC4S3A in palmitate-treated HCs was abrogated when the cells expressed 3KR-DACH1 instead of WT DACH1 (Figure 7F, bottom two blots). These combined results show that nuclear HDAC4 decreases DACH1 through SUMOylation and subsequent proteasome-mediated degradation. With this finding, we present a summary of the pathway in which CaMKII activation in HCs in obesity leads to defective insulin signaling (Figure 7G).

DISCUSSION

Understanding how ATF6 is suppressed in HCs in obesity is a critical issue in view of its importance in both insulin signaling, supported by our previous study (Ozcan et al., 2013) and bolstered here, and HGP (Wang et al., 2009). Additionally, two independent studies reported associations between variants of the *ATF6* gene in humans and disturbed glucose homeostasis and T2D (Meex et al., 2007; Thameem et al., 2006). The mechanism of ATF6 suppression revealed here involves a very interesting pathway in which the half-life of the corepressor DACH1 is prolonged, leading to decreased transcription of the *Atf6* gene. Even though the key step in ATF6 activation is its post-translational proteolytic cleavage and nuclear translocation (Ye et al., 2000), the data here imply that the level of *Atf6* transcription becomes rate limiting in HCs in the setting of obesity.

Our work shows that decreased levels of nuclear HDAC4 in HCs in obesity indirectly lowers *Atf6* transcription by increasing the level of DACH1. In this context, a recent paper using an unbiased proteomics approach found that obesity in humans is associated with a decrease in HDAC4, which improves with physical exercise, suggesting the possibility that nuclear HDAC4 is protective in obesity (Abu-Farha et al., 2013). In contrast, Mihaylova et al. reported that combined silencing of hepatic HDAC4, 5, and 7 increases FoxO1 acetylation in fasted lean mice and lowers HGP in lean and obese mice (Mihaylova et al., 2011). In this regard, we found no difference in the level of acetylated FoxO1 in the livers of obese mice treated with constitutively nuclear HDAC4 or HDAC4 shRNA. Moreover, while the major endpoint of the Mihaylova et al. study was blood glucose levels, the study here focused on liver insulin signaling and whole-body insulin sensitivity.

HDAC4, like other class IIa HDACs (5, 7, and 9), exhibits very low deacetylase activity because of the substitution of a catalytic Tyr with His (Lahm et al., 2007). As such, the role of HDAC4 shown here is related its ability to SUMOylate and thereby promote the proteasomal degradation of DACH1 when HDAC4 is nuclear, e.g., in the lean state. Previous studies have shown that HDAC4 can serve as SUMO E3 ligase for a number of target proteins in various cell types (Ghisletti et al., 2007; Grégoire and Yang, 2005) and that SUMOylation can serve as a priming process for ubiquitination, leading to protein ubiquitination and proteasomal degradation of the target proteins (Lee et al., 2014; Liu et al., 2013; Miteva et al., 2010). Consistent with the findings here, only the nuclear form of HDAC4 possesses SUMO E3 ligase activity (Lee et al., 2009; Yao and Yang, 2011). Of note, DACH1 can be affected by other post-translational modifications that affect its function, including acetylation and phosphorylation

(Chen et al., 2013; Wu et al., 2014), and thus it will be interesting to examine in the future whether these modifications of DACH1 occur in HCs in obesity.

Class IIa HDACs can affect gene transcription by other mechanisms, but none of these appear to be involved in the pathway described here. For example, HDAC4 can effect gene repression by recruiting HDAC3 and its coregulators to the nucleus, which repress gene transcription (Fischle et al., 2002). However, we found that siRNA mediated-silencing of HDAC3 did not suppress *Atf6* transcription in palmitate-treated CaMKII-deficient HCs (data not shown). Consistent with the lack of a role of HDAC3 in the pathway presented here, a recent study showed that when HDAC3 is silenced in HCs in obese mice, metabolic precursors are rerouted away from glucose production into lipid synthesis, causing steatosis and improved insulin sensitivity (Sun et al., 2012).

Most studies on DACH1 have focused on its roles in development and tumorigenesis (Popov et al., 2010). Ironically, one of the developmental roles is related to perinatal pancreatic β cell proliferation, which is associated with DACH1-mediated repression of p27Kip1 (Kalousova et al., 2010). The hypothesis that DACH1 is required for normal insulin secretion was suggested as a mechanism to explain a link between a *DACH1* polymorphism and diabetes in a Chinese population (Ma et al., 2014), but replication in additional populations and direct links to β cell function are needed to substantiate this claim. In contrast, the findings here show a detrimental, non-developmental role of liver DACH1 in obesity-associated insulin resistance. Accordingly, silencing of DACH1 in the livers of obese mice, by improving insulin signaling in HCs and improving overall metabolism, corrects hyperinsulinemia. Moreover, we found a striking correlation between hepatic DACH1 levels and BMI in humans.

We provide evidence that suppression of *Atf6* in HCs in obesity involves a DACH1-NCOR complex. However, this finding does not imply that hepatic NCOR silencing in obese mice would mimic the metabolic improvement seen with hepatic DACH1 silencing, because NCOR has functions in liver that are independent of DACH1. For example, NCOR in complex with HDAC3 represses genes involved in hepatic lipogenesis, and thus one of the effects of HC-targeted NCOR deletion is steatosis (Sun et al., 2013). In contrast, hepatic steatosis is improved by liver-targeted silencing of DACH1 (not shown) or its upstream effector, CaMKII (Ozcan et al., 2013).

In summary, our study reveals a role for DACH1 in obesity-induced glucose intolerance and insulin resistance in mice, with a striking correlation between liver DACH1 level and obesity in humans. While DACH1 upregulation in obesity may affect a number of different genes and pathways, its ability to suppress insulin signaling by repressing *Atf6* transcription appears to be dominant. Nonetheless, future studies will likely reveal additional DACH1 targets in HCs that may shed additional light on gene expression changes in obesity. Finally, we recently published that drug-mediated inhibition of MK2, a kinase downstream of CaMKII in HCs in obesity, improves metabolism in obese mice (Ozcan et al., 2015), which is consistent with genetic studies conducted by both our group (Ozcan et al., 2013) and an independent laboratory (Ruiz et al., 2016). Given that the upstream kinase pathway promotes diabetes by suppressing ATF6, the findings in this report provide important mechanistic underpinnings for

future therapeutic strategies that attempt to improve metabolism in obese T2D subjects by targeting this pathway.

EXPERIMENTAL PROCEDURES

Mouse Experiments

ob/ob and DIO mice were obtained from The Jackson Laboratory. DIO mice were fed a high-fat diet with 60% kcal from fat (Research Diets) and maintained on a 12-hr light-dark cycle. *Dach1^{fl/fl}* mice were generated as described previously (Chen et al., 2015). The detailed procedures are shown in Supplemental Experimental Procedures.

Primary HCs

Primary mouse HCs were isolated from 8- to 12-week-old mice as described previously (Ozcan et al., 2012). Unless indicated otherwise, the cells were cultured in DMEM containing 10% fetal bovine serum, treated as described in the figure legends, and then incubated for 5 hr in serum-free DMEM before harvesting. The detailed procedures are shown in Supplemental Experimental Procedures.

Statistical Analysis

All results are presented as mean \pm SEM, and p values were calculated using the Student's t test for normally distributed data and the Mann-Whitney rank sum test for non-normally distributed data. One-way ANOVA with post hoc Tukey test was used to evaluate differences among groups when three or more groups were analyzed.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.05.006>.

AUTHOR CONTRIBUTIONS

L.O. and I.T. designed the research. L.O., D.S.G., Z.Z., and J.C.d.S. conducted the research. L.O., D.S.G., Z.Z., J.C.d.S., K.C., M. Bessler, M. Bagloo, B.S., R.P., and I.T. analyzed the data. L.O., R.P., and I.T. wrote the manuscript. K.C. and R.P. contributed new reagents and analytical tools. M. Bessler, M. Bagloo, and B.S. organized patient recruitment and human liver sample collection.

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